DETERMINING PATERNITY IN LOGGERHEAD TURTLE (*Caretta caretta*) NESTS ON MELBOURNE BEACH, FLORIDA USING MICROSATELLITE MARKERS

by

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ABSTRACT

Many aspects of sea turtle biology are difficult to measure in these cryptic migratory species, and this undercoverage continues to hamper conservation efforts. One such parameter is the health and size of breeding populations; generally it is not known how many males contribute to the next generation. Allozyme analysis suggested multiple paternity in loggerhead turtle clutches in Australia, and subsequent studies indicated that the frequency of multiple paternity varies from species to species and perhaps location to location. This study examined fine-scale population structure and paternal contribution to loggerhead clutches on Melbourne Beach, FL, USA using microsatellite markers. Mothers and offspring from 70 nests collected at two locations were analyzed using two to four polymorphic microsatellite loci. Fine-scale population structure was not evident between the sampled beaches. Multiple paternity was common in loggerhead nests on Melbourne Beach; of 70 clutches analyzed, 22 had more than one father, and 6 had more than two fathers. This is the first time that more than two fathers have been detected in sea turtle nests.

INTRODUCTION

I used molecular techniques to investigate whether loggerhead turtle clutches deposited on Melbourne Beach, Florida were fertilized by multiple males. I also investigated whether population substructure existed within this population on a very small scale (about 10 km), similar to that reported for green turtles on Tortuguero, Costa Rica (Peare and Parker, 1996).

Loggerhead turtles are listed as threatened under the U.S. Endangered Species Act of 1973, and are restricted from international trade by Appendix I listing in the Convention on International Trade in Endangered Species (CITES). As a result, a recovery plan has been formulated for the species. Item 2211 in the *Recovery Plan for US Population of Loggerhead Turtle Caretta caretta* (National Marine Fisheries Service and U. S. Fish and Wildlife Service, 1991) cites the need to "Determine seasonal distribution, abundance, population characteristics, and status in inshore and nearshore waters." Most estimates of population size and health are based on numbers of nesting females (Murphy and Hopkins, 1984; Ehrhart, 1989), which can be counted with beach patrols. It is harder to generate estimates of the number of reproductive males contributing to the next generation.

The number of reproductive males directly affects N_e, the effective population size (Nunney, 1993; Waite and Parker, 1997). As N_e decreases, loss of genetic diversity is accelerated, so a population may have diminished ability to adapt and survive in the face

of environmental change. Small populations are particularly sensitive to changes in the numbers of mating males because genetic drift can act more rapidly in these populations to eliminate rare alleles.

The world's second-largest nesting concentration for the loggerhead sea turtle is in central Florida in the southeastern U. S. (Ross, 1982). In the summer, gravid females lumber onto these beaches to dig nest cavities and deposit eggs in the warm sand. Females may return throughout the summer to deposit up to seven clutches (Hirth, 1980; Talbert *et al.*, 1980; Dodd, 1988) before returning to the sea for 2–3 years, after which they often return to the same beach to nest (Bjorndal *et al.*, 1983; Dodd, 1988; Van Buskirk and Crowder, 1994).

After 50–60 d of incubation, hatchlings emerge at night in late summer and scramble for the light horizon of the water (Dodd, 1988). They apparently spend their "lost years" of post-hatchling life at sea, floating among rafts of *Sargassum* or along convergence zones of the North Atlantic for an undetermined period of time (Fletemeyer, 1978; Carr, 1987; Witherington, 1994). Juveniles reappear near shore some years later, at a curved carapace length of 30–50 cm (Musick and Limpus, 1997). Aerial surveys, tracking and mark-recapture studies in the western North Atlantic demonstrate that juveniles, sub-adults, and adults occupy near-shore foraging grounds with high site fidelity (Keinath, 1993; Musick and Limpus, 1997). When they reach maturity 10–30+ y after hatching (Mendonça, 1981; Frazer, 1983; Frazer and Ehrhart, 1985; Klinger and Musick, 1995), females begin migrating to mating and nesting grounds.

Mark-recapture data and radio and satellite tagging indicate that the time between nesting seasons is spent on near-shore foraging grounds or in transit between foraging and

mating/nesting beaches (Musick and Limpus, 1997). Mating is believed to occur primarily in breeding aggregations near nesting beaches (Dodd, 1988). While adult females regularly return to their natal beaches to nest, it is not known whether males also make philopatric migrations to mate, or whether resident males mate opportunistically with females passing through feeding grounds on their way to the beach (Dodd, 1988; FitzSimmons, 1997; Miller, 1997). Capture and stranding data, however, indicate that male turtles increase in abundance in the inshore waters of the southeastern U. S. preceding the nesting season (Henwood, 1987; Sally Hopkins-Murphy, pers. comm.), and recent aerial observations of mating loggerheads suggest that mating occurs near the rookeries (Frick *et al.*, 2000). However, this does not prove that males return to their natal region to father the next generation of hatchlings (but see FitzSimmons *et al.* 1997 for a case of male philopatry in green turtles).

Most knowledge of sea turtle biology has been gleaned from the small fraction of life they spend on shore, and from radio, satellite and mark-recapture studies. Because of the small size of sea turtle hatchlings, the high mortality at the hatchling stage [only one in 1000 is estimated to reach maturity (Frazer, 1986)], and their indeterminate growth, it is impractical to tag hatchlings and follow them to adulthood. Adult sea turtles are difficult to study when in the water, and tags applied when they are on land or hauled into boats are often shed within a few years (Frazer, 1983; Limpus, 1992; Bjorndal *et al.*, 1996).

Long-standing hypotheses about sea turtle life histories which could not be tested using traditional tag-return and tracking techniques can now be examined using molecular techniques. For example, molecular evidence supports female philopatry in nesting green

(Bowen *et al.*, 1992; Allard *et al.*, 1994; Norman *et al.*, 1994), loggerhead (Bowen *et al.*, 1994) and hawksbill turtles (Broderick *et al.*, 1994; Bass *et al.*, 1996). However, these studies were designed to test for philopatry on a regional scale, and the true precision of natal homing has yet to be determined. Mediterranean loggerheads exhibited population structure on a scale of about 100 km. This structure existed in both the nuclear and mitochondrial genomes, indicating restricted male- and female-mediated gene flow (Schroth *et al.*, 1996). Peare and Parker (1996) found evidence of fine scale geographic population structure in green turtles nesting at Tortuguero, Costa Rica. Tortuguero green turtles that nested closer together were more closely related than those that nested several miles apart, a testimony to the precision of natal homing in Tortuguero green turtles. However, Peare and Parker (1996) could not replicate these results with green turtles in Melbourne Beach, FL.

Using mixed stock analyses, juvenile and adult turtles at feeding aggregations have been linked with their corresponding rookeries (Broderick *et al.*, 1994; Sears *et al.*, 1995; Bagley, 2000). Trans-Pacific migrations were documented by linking haplotypes in turtles around Baja California to those on Japanese nesting beaches (Bowen *et al.*, 1995). More recent tag return and tracking information have verified these findings (Resendiz *et al.*, 1998). In addition, the genetic composition of rookeries (Bowen *et al.*, 1992; Allard *et al.*, 1994; Broderick *et al.*, 1994; Norman *et al.*, 1994; Bass *et al.*, 1996; Encalada *et al.*, 1996) and male contributions to gene flow (Karl *et al.*, 1992; FitzSimmons *et al.*, 1995; FitzSimmons, 1997) were examined using microsatellites, sequencing, denaturing gradient gel electrophoresis, and restriction fragment length polymorphism analysis of nuclear and mitochondrial loci.

Studies of paternity in sea turtles have been limited by small sample sizes and/or ambiguous markers. It is not apparent whether multiple paternity is the exception or the rule among sea turtles. Harry and Briscoe (1988) used allozymes to infer that at least some loggerhead clutches in Queensland, Australia, had multiple paternity, and the presence of multiple fathers has been suggested in a preliminary study of green turtle clutches at Tortuguero, Costa Rica (Peare, 1994; Parker *et al.*, 1996). Kemp's ridley clutches at Rancho Nuevo, Mexico have a high rate of multiple paternity (Kichler, 1996). Bollmer *et al.* (1999) found that one of three loggerhead clutches from Melbourne Beach, FL, had multiple fathers, with two fathers more likely than three. However, this study used 20 cm polyacrylamide gels in lieu of sequencing gels, and adjacent microsatellite alleles might not have been successfully resolved. On the other hand, Rieder *et al.* (1998) and Dutton and Davis (1998) found no evidence of multiple paternity in leatherback nests in Costa Rica or St. Croix, and FitzSimmons (1998) found a very low incidence of multiple paternity in green turtles nesting in the southern Great Barrier Reef.

Polyandry is costly in terms of energy, time, added danger of predation, and mating-induced damage (Jennions and Petrie, 2000). Though monogamy has a lower energetic cost, lower injury and predation risk, and lower risk of disease transmission, sea turtles are unlikely to be monogamous. In species that are monogamous or socially monogamous, there is often high parental investment in a few, demanding young (review: Birkhead and Møeller, 1992; Negro *et al.*, 1996; Birkhead, 1998; Runcie, 2000). Sea turtles, however, do not pair-bond and provide no parental care to their many young. Other hypothesized benefits of monogamy include, but are not limited to: access to males' territory (and resources contained therein) and protection from other males (Emlen

and Oring, 1977; Kleiman and Malcolm, 1981; Quinn *et al.*, 1999). Monogamy is also thought to be adaptive if potential mates are rare (Reavis and Barlow, 1998), or limited resources are uniformly distributed (Emlen and Oring, 1977). Again, sea turtles do not seem likely to gain from monogamy in most of these scenarios: males do not defend territories so that females can forage in them, males certainly do not appear to protect their mates from other males (Booth and Peters, 1972), and foraging grounds in most species are patchily distributed along shorelines. There is, however, a chance that males could be limiting at Melbourne Beach, FL, as the sex ratio of hatchlings leaving the beach is more than 9:1 female:male (Mrosovsky and Provancha, 1989).

Loggerheads are likely to be promiscuous, as Peare (1994) proposed for green turtles. In a promiscuous system, the genetic contributions of fathers of unsuccessful clutches of eggs will persist in clutches deposited by successful females, and only the mother's genetic contribution will be lost with the unsuccessful clutch. This suggests that, if most males mate successfully with a number of females, more genetic diversity would be maintained in a promiscuous than a monogamous system, providing hope for the future recovery of genetically depauperate species (Peare, 1994; Sugg and Chesser, 1994). In a sea turtles, however, this, is not the strongest argument for polyandry, since females typically lay several nests over a season, and it is unlikely that they would all be unsuccessful (except in the case of catastrophe such as a hurricane, in which case it is likely that all nests would be lost, including other nests with the fathers' genes). It is more likely that female loggerheads would derive other genetic benefits from multiple matings. These benefits could include bet-hedging (Watson, 1991; Watson, 1998), avoidance of genetic incompatibility (reviewed in Zeh and Zeh, 1996; Newcomer *et al.*, 1999),

avoidance of inbreeding (Madsen *et al.*, 1992; Stockley *et al.*, 1993), gaining "good genes" (Keller and Reeve, 1995), and increased genetic diversity among offspring (Watson, 1991; Madsen *et al.*, 1992; Baer and Schmid-Hempel, 1999).

For sea turtles to benefit from bet-hedging, there must be variability in heritable fitness traits in male turtles, and it may also be difficult for females to accurately assess the quality of a potential mate (Jennions and Petrie, 2000). Because a female may not be able to tell if her choice of a potential mate is good, she may mate with several males in order to reduce the variance in male quality and hedge her bets against low-quality mates (Watson, 1991; Watson, 1998). Alternatively, a female who mates with a convenient male early in the season may later encounter a more desirable male and mate again to "upgrade" from her earlier choice (Birkhead *et al.*, 1993; Evans and Magurran, 2000).

Genetic incompatibility could stem from a number of causes, including cellular endosymbionts, transposons, genomic imprinting, segregation disorders, and maternal-effect lethals (reviewed in Zeh and Zeh, 1996). These selfish genetic elements and their host organism(s) are often at odds as they attempt to reproduce. This interaction between the nuclear and cytoplasmic genomes of a particular mating pair of turtles could have a negative effect on the female's fitness, and polyandry could protect a female against a reduction in viable offspring due to a genetically incompatible mate (Zeh and Zeh, 1996).

The quest for "good genes" is explained by Keller and Reeve's (1995) sexually selected sperm hypothesis. In this scenario, females mate multiply, and the males with the most competitive sperm secure paternity. Assuming that the competitive superiority of the successful sperm is heritable, the sons of polyandrous females will have sperm that is superior to that of the sons of monandrous females.

Genetic diversity provides females insurance against an uncertain future in a changing environment (Smith, 1984). Increased genetic diversity among offspring has also been shown to reduce parasite loads in eusocial insects (review: Schmid-Hempel, 1998; Baer and Schmid-Hempel, 1999).

I used highly variable microsatellites amplified by the polymerase chain reaction (PCR) to determine: 1) whether clutches of loggerhead turtle eggs from Melbourne Beach, FL were fertilized by more than one male, and 2) if population substructure existed within 8 km on this nesting beach.

Microsatellites are most often dinucleotide repeats found in nuclear DNA (such as CACACACA...), which have high mutation rates due to slippage events in DNA synthesis (Schlötterer and Tautz, 1992). Microsatellites used in population studies are usually highly variable in length (number of repeats). Due to this high mutation rate and the biparental inheritance of nuclear DNA, microsatellites are well-suited for studies of paternity and pedigree (Tautz, 1989). Individuals have unique complements of alleles across several loci, which can be resolved on polyacrylamide sequencing gels. PCR technology enables one to target and amplify a specific region of DNA with primers that anneal to short sequences flanking the region of interest. This allows analyses to be conducted from small amounts of blood, nest salvage materials (egg remains and undeveloped embryos), and small, nonlethal tissue biopsies from hatchlings. Nonlethal retrieval of DNA from hatchlings allowed collection of large sample sizes without adversely affecting this threatened species. The primers I initially used in this study (Cc141, Cc7, Ei8, and Cm72) were developed for use in marine turtles (FitzSimmons *et al.*, 1995; FitzSimmons, 1997). To validate the results from these markers, I used two

additional loci, CCM2 (Francisco[1], unpublished data) and Ccar176 (this study). These markers collectively provide evidence of multiple paternity in Melbourne Beach loggerheads.

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MATERIALS AND METHODS

Sampling from nesting females and marking of nests

I chose two sampling locations on Melbourne Beach, based on nest density and accessibility. Each site covered about 1.5 km of beach, with one immediately north of the Archie Carr National Wildlife Refuge (28" 2.3" N, 80" 32.6" W), and the other about 8 km south, within the Refuge (27" 57.7" N, 80" 30.3" W; hereafter referred to as the northern and southern sites). Blood samples were collected from 75 nesting loggerheads at each site during June 9-22, 1996. After the female deposited eggs, sand was removed to create a shallow cavity into which her head would droop, and preservative-free 3 ml draw Vacutainers (Becton, Dickenson, and Co., Forest Lakes, NJ) fitted with sterile 21 gauge needles were used to collect l-2 ml of blood from the dorsal cervical sinus [after the method of Owens and Ruiz (1980)]. Blood was immediately poured from the Vacutainers into tubes containing 10 ml SDS-urea buffer, pH 6.8 (1% SDS, 8 M urea, 240 mM Na_2HPO_4, 1 mM EDTA).

Nests were marked in a manner similar to that used by the University of Central Florida's Marine Turtle Research Group (Dean Bagley[2], pers. comm.; Ehrhart and Witherington, 1986). After blood was drawn and the nesting female finished covering her nest, the egg chamber was located by gently digging, thoroughly recovered, and then

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marked with a temporary stake directly over the center of the chamber. Two stakes were labeled with the nest number and the date of deposition, and placed in the dunes in a straight line directly landward of the nest site. One stake was hidden, and the other was obvious. The distance from each stake to the nest was then measured and recorded. Aluminum cans were also buried in the sand 0.5 m to the north and south to facilitate finding the nest later using a metal detector. Finally, the temporary stake directly over the egg chamber was removed, and the sand above the egg chamber was rearranged to prevent predators from detecting it. The nest location was then recorded after pacing from a nearby landmark.

Sampling hatchlings

Hatchlings were sampled July 2%August 17, 1996, beginning approximately 50 d after the first nest was laid. Nests were located by measuring from the stakes and surveying with a metal detector. Nest locations were verified by hand-digging to the top of the egg chamber, and hardware cloth cages were used to detain emergent hatchlings and deter predators (Fig. 1). A seaward door at the base of the cages allowed hatchlings to escape if they emerged during the day. Cage doors were shut at dusk, opened at dawn, and checked at least twice nightly for emergent hatchlings.

Up to 20 hatchlings from each nest were sampled by biopsying the shell with a 2 mm disposable punch (Fray Products, #BP20, Buffalo, NY). The biopsy site was a rear marginal scute, tangent to the edge of the shell (Fig. 2). To ensure that hatchlings were chosen randomly, one person counted the hatchlings into a canvas bag, and then another person chose up to 20 numbers from a list of random numbers. As hatchlings were taken

out of the bag and placed on the sand, the sampler retained those designated by the random numbers. Hatchlings were sampled from all emergences. No more than 20 hatchlings from each clutch were sampled. Biopsies were stored in 70% ethanol until DNA extraction. Unsampled hatchlings were released as they were counted, and others were released immediately after sampling. All sampling was in accordance with Florida Department of Environmental Protection Marine Turtle Permit 021.

Isolating DNA from shell

I used standard protocols to extract DNA from hatchling shell biopsies (Palumbi *et al.*, 1991). Shell biopsies were rinsed with sterile water, dried briefly in a 37 °C oven to evaporate remaining ethanol, and digested at 56 °C overnight in 0.25 ml proteinase K buffer (0.5 µg proteinase K per µl of digestion buffer: 100 mM EDTA, 10 mM Tris pH 7.5, 1% SDS). I then isolated the DNA by extracting twice with phenol:chloroform:isoamyl alcohol (PCI; 25:24:1) followed by one extraction with chloroform:isoamyl alcohol (CI; 24:1). Immediately following CI extraction, 1 µl of oyster glycogen (20 µg/µl) and 0.1 volume of 3 M sodium acetate pH 5.5 were added to the aqueous solution, and DNA was precipitated with either an equal volume of cold 100% isopropanol or 2 volumes of cold 100% ethanol (Sambrook *et al.*, 1989; Ausubel *et al.*, 1994). I then pelleted the DNA by centrifugation at 4 °C for 20 min at 30,000 x g. Pellets were washed with 70% ethanol, dried under vacuum (Speedvac, Savant Instruments, Holbrook, NY), and rehydrated in 1× TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). DNA yields were quantified with a fluorometer (Hoefer DyNAQuant 200, San

Francisco, CA), and ranged from below detection up to 120 µg per biopsy, with most samples yielding 10–40 µg of DNA.

Isolating DNA from blood

DNA from 200–400 µl of blood diluted in SDS-urea was extracted twice with PCI followed by two extractions with CI. The aqueous phase was then dialyzed against water overnight at 4 °C (White and Densmore, 1992, protocol 11). DNA was ethanol-precipitated as above when sample volumes were < 0.5 ml, or precipitated by n-butanol extraction if > 0.5 ml. Centrifugation followed precipitation, and the resultant pellet was rinsed with cold 70% ethanol, dried, and re-hydrated in 50–250 µl 1× TE. DNA yields were quantified with a fluorometer (Hoefer DyNAQuant 200, San Francisco, CA), and ranged from 10–100 µg per sample, with most samples yielding 40–60 µg of DNA.

Developing microsatellite primers

Construction of DNA library

I constructed a microsatellite-enriched DNA library to isolate and sequence putative microsatellite clones from which to design primers (based on Armour *et al.*, 1994, and modified by the University of Florida Education core). Bold letter designations on the following steps correspond to letters in Fig. 3: **A)** I pooled loggerhead DNA from several animals, digested approximately 20 µg of this DNA overnight with *Sau* 3AI (New England Biolabs #169), and then **B)** ran the digestion products on a 2% NuSieve agarose (BioWhittaker Molecular Applications, Rockville, MD) gel containing 0.1 µg/ml ethidium bromide and 1× Tris-acetate-EDTA (TAE: 0.4 M Tris-acetate, 0.001 M EDTA).

The region spanned by 400–800 base pair (bp) markers was excised using a clean single-edged razor blade, and digested overnight with 10 U agarase (#A6306, Sigma, St. Louis, MO). **C)** DNA was purified from the digested band by extracting it first with an equal volume of Tris-equilibrated phenol, pH 8.1, followed by a PCI and a CI extraction. **D)** DNA was precipitated with the addition of 0.1 volume of 3 M sodium acetate, pH 5.2, 40 µg oyster glycogen, and 1 volume of isopropanol (4 °C for 4 hours), and then pelleted by centrifugation. The pellet was rinsed with 70% ethanol and dried under vacuum before rehydrating it in 100 µl 1× TE.

I prepared linkers from the oligonucleotides Sau-L-A (5′ - GCG GTA CCC GGG AAG CTT GG - 3′) and Sau-L-B (5′ - GAT CCC AAG CTT CCC GGG TAC CGC - 3′; Armour *et al.*, 1994) as follows (bold number designations on the following steps correspond to numbered steps in Fig. 3): **1)** T4 polynucleotide kinase (T4 PNK; New England Biolabs #201) was used to phosphorylate Sau-L-B in a 50 µl reaction containing 5 µg Sau-L-B, 1× provided kinase buffer, 10 mM ATP, and 20 U T4 PNK. The reaction incubated at 37 °C for 30 min, and then the T4 PNK was heat denatured for 20 min at 65 °C. **2)** 5 µg Sau-L-A in 50 µl of 1× kinase buffer was added to the phosphorylated Sau-L-B, and the oligonucleotides were annealed by heating the mixture for 2 min at 85 °C, 15 min at 65 °C, 15 min at 37 °C, 15 min at room temperature, and 15 min on ice. **3)** I then extracted the annealed linker solution once with 200 µl PCI, centrifuged it briefly to separate the phases, and transferred the aqueous layer to a clean tube. **4)** Linkers were precipitated by adding 11 µl 3 M sodium acetate (pH 5.2) and 333 µl 100% ethanol and storing this mixture on ice for 30 min. Linkers were pelleted by centrifugation at

13,600 rpm for 20 min at 4 °C, ethanol was removed by pipetting, and the pellet was rinsed with 500 µl cold 70% ethanol and dried under vacuum before being resuspended in 10 µl sterile water. **5)** Linkers were then ligated to the purified, size-selected turtle genomic DNA fragments in a 20 µl reaction containing 4 µl Sau linker solution, 200 ng digested and size-selected genomic DNA, 1× ligase buffer, 0.01 mg/ml BSA, and 0.8 U T4 DNA ligase (New England Biolabs, #202). I incubated the mixture overnight at 16 °C, and **6)** gel-fractionated the resulting ligated DNA on a 1.5% NuSieve gel to remove unligated linkers (again selecting the 400–800 bp region). DNA was purified from the gel slice as above, and then **7)** amplified with Sau-L-A. PCR conditions for four 100 µl reactions were as follows: 1× PCR buffer, 2.5 mM $MgCl_2$, 0.2 mM dNTPs, 1 µM Sau-L-A, 5 U Taq DNA polymerase (Gibco Life Technologies, Rockville, MD, #10342), and 4–6 µl cleaned and linker-ligated DNA. Cycling parameters consisted of an initial denaturation at 94 °C for 3 min, followed by 25 cycles of 45 s at 94 °C, 45 s at 68 °C, and 1.5 min at 72 °C. The last cycle was followed by a final 5 min extension at 72 °C. **8)** I then pooled PCR products, purified them using a Qiaquick PCR Purification Kit (Qiagen, Valencia, CA, #28104), and eluted them in 80 µl 10 mM Tris-HCl, pH 8.5.

Enrichment of DNA library

I enriched the newly-constructed DNA library largely as described in Karagyozov *et al.* (1993; modified by the University of Florida Education Core). I spotted 2.0 µg of each of several different oligonucleotides dissolved in H_2O (AAT_8, ACG_8, AGG_8, AAG_8, GT_{30}, GGT_8, $GTGA_8$) onto separate 0.5 cm diameter circles of sterile Hybond+ nylon membrane. The membranes were air-dried, rinsed in water, baked for 2 h at 80 °C, and

then cross-linked for 2.5 min. Membrane filters were then washed in 1.5 ml hybridization solution [50% formamide, 5 × SSC (0.75 M NaCl, 0.75 M sodium citrate pH 7.2), 50 mM Na_2PO_4, 7% SDS] for 48 h at 37 °C, followed by a 10 min wash in 1% SDS in a boiling water bath to remove unbound oligonucleotides. Filters were then air-dried and stored at 4 °C.

I hybridized 20 µg of heat-denatured PCR-amplified library fragments to the filters for 48 h at 37 °C with 600 µl of hybridization solution and in the presence of 10 µg of the Sau-L-A primer, to prevent the oligonucleotides' complementary ends from concatemerizing. I followed the hybridization with a series of washes: 1) with wash buffer (2 × SSC, 1% SDS, 50 mM Na_2PO_4) for 30 min at 37 °C; 2) with 1× SSC for 30 min at 37 °C; and 3) with 0.1× SSC for 30 min at 65 °C. I then eluted DNA from the filters in 0.5 ml 1% SDS for 3 min in a boiling water bath. DNA was precipitated with 40 µg oyster glycogen, 200 µl 5 M LiCl, and 1 ml 95% ethanol overnight at -20 °C. DNA was pelleted by centrifugation for 10 min at 30,000 x g, then the pellet was washed with 70% ethanol, dried at room temperature for 1 h, and resuspended in 30 µl Milli-Q water. I used 4 µl of this DNA as the template in each of four 100 µl PCR reactions with the Sau-L-A primer (same conditions as previous Sau-L-A PCR, above). PCR products were purified using a Qiaquick PCR Purification Kit, pooled, and quantified using a fluorometer. The yield after purification and pooling was 24 µg DNA. All of this PCR product was then heat-denatured and used in a second hybridization to the previously-used filters, followed again by PCR with Sau-L-A, as outlined above. About 3 µg of the final PCR product was then digested overnight with *Sau* 3AI, and the digested DNA was cleaned with a Qiaquick PCR Purification Kit.

I ligated library fragments into pUC 19 vector in a 10 µl reaction containing 85 ng insert DNA, 300 ng BAM/BAP pUC 19 vector (Amersham Pharmacia Biotech #27-4855-01), 0.8 U T4 DNA ligase, and 1× ligase buffer. Ligation was performed at 16 °C overnight, and ligase was subsequently inactivated by heating to 65 °C for 10 min. I diluted the ligation mix 1:5, and transformed four 100 µl aliquots of DH5α competent *Escherichia coli* cells (Gibco #98258) with 1–5 µl of the ligation product as indicated in the manufacturer's instructions. I then plated transformed cells onto LB agar containing 50 µg/ml carbenicillan and treated with X-Gal (5-Bromo-4-chloro-3-indoxyl-beta-D-galactopyranoside). Plates were incubated overnight at 37 °C and then refrigerated to intensify the blue color in colonies without inserts. I amplified inserts from positive (white) colonies by lightly touching each colony with a sterile pipette tip, and then gently rinsing the pipette tip in 50 µl of PCR cocktail [0.6 mM each of M13 -40 and -24 primers (New England Biolabs, Beverly, MA, #1212 and #1201, respectively), 2 mM $MgCl_2$, 2.5 U Taq DNA polymerase, 0.2 mM each dNTP, and 1× PCR buffer] in a 0.2 ml reaction tube. Cycling parameters consisted of an initial denaturation at 94 °C for 3 min, followed by 35 cycles of 45 s at 94 °C, 45 s at 53 °C, and 20 s at 72 °C, with a final 5 min extension at 72 °C. I treated amplified products with 10 µl RNase A (100 µg/ml) to remove RNA contaminants, electrophoresed them on 1% agarose gels containing ethidium bromide, and visualized them under fluorescence. PCR products larger than 300 bp were purified by electrophoresis in low-melting point agarose. Bands were excised, melted at 65 °C, and subjected to digestion with 5 U agarase overnight at 41 °C. I then used 2–8 µl of this digestion in a cycle sequencing reaction using fluorescently labeled di-deoxy terminators according to the manufacturer's recommended conditions

(Applied Biosystems, Inc. Big Dye Terminator Cycle Sequencing Ready Reaction Kits, #4303152). I ethanol-precipitated and dried sequencing products before resuspending and analyzing them on an ABI 310 automated sequencer (Applied Biosystems, Foster City, CA). Over 300 clones were sequenced, and many revealed long (> 400 bp) imperfect repeats, repeat sequences that were microsatellites within minisatellites, or microsatellites of a desirable size, but with insufficient flanking sequence for primer design.

Using the computer program Primer3 (Rozen and Skaletsky, 1998) and the Operon oligo toolkit web page (www.operon.com/toolkit), I designed primers from unique loci containing six or more repeat units and flanked by 20 or more bp of unique, non-repetitive sequence.

Because *Taq* polymerase often adds a non-templated adenine at the end of a replicated strand of DNA, microsatellite PCR products can have additional bands at 1-bp intervals from the non-adenylated allele, which can make it difficult to decide which peak to score. To achieve consistent scores, plus-a product formation was encouraged by PIGtailing primers by adding 2–4 non-templated bases on the 5' end of one primer (as described in Brownstein *et al.* 1996). Encouragement of the plus-a product not only decreased the signal of non-adenylated products, but also increased the signal of the adenylated alleles that I scored. Primers were synthesized by Operon Technologies, Inc. (Alameda, CA), with one primer of each pair labeled with a fluorescent phosphoramidite dye. Primers were designed for 11 microsatellites; nine amplified successfully, with seven monomorphic in the four individuals tested. Ccar176, one of the remaining two polymorphic microsatellites, amplified well and consistently (Fig. 4). The other locus, (Ccar199) stuttered too badly to score adjacent alleles reliably (Fig. 4), and was not used.

Stutter is thought to occur because of *Taq* polymerase slippage during replication of repetitive areas of DNA (Schlötterer and Tautz, 1992), and is particularly prominent in dinucleotide microsatellites (Armour *et al.*, 1999). Stutter appears as a ladder of bands (or series of peaks, in the case of electropherograms) that are one repeat unit apart and almost always smaller in size than the true allele. (see Appendix A for primer sequences and annealing temperatures).

Initially, I PCR-amplified microsatellites for the mothers with radiolabeled primers Cc7, Cc141 (FitzSimmons, 1997), Ei8, and Cc117 (FitzSimmons *et al.*, 1995). I chose the two most polymorphic loci (Cc7 and Cc141) to amplify at least ten offspring from about half of the clutches. When these two loci produced ambiguous results concerning multiple paternity, I used locus Ei8. Radiolabeled PCR products were resolved on denaturing 6% acrylamide sequencing gels, and alleles were scored based on comparisons with a sequenced size standard. Mothers were run alongside their offspring. Offspring that yielded unexpected alleles were re-amplified and re-run alongside their mother to confirm results. With these three loci, it was still not always apparent whether "extra" alleles (alleles that might represent a second father) that were present only at one or two loci resulted from multiple paternity or mutation, so I used loci CCM2 (Francisco, unpublished data) and Ccar176 (this study) for further analyses. All mothers and at least ten offspring for each clutch were assayed at these two loci. I amplified samples in 25 µl reactions containing 25–50 ng of turtle genomic DNA, 1× Gibco PCR buffer, 0.12 mM each dNTP, 1.2 mM $MgCl_2$, 0.35 U Gibco Taq, 0.25 mg BSA, and 240 nM each primer. Cycling parameters consisted of an initial denaturation at 94 °C for 2 min, followed by 30 cycles of 30 s at 94 °C, 30 s at an optimal annealing temperature (58 °C for CCM2; 60 °C

for Ccar176), and 45 s at 72 °C, followed by a final 5 min extension at 72 °C. One primer of each pair was labeled with either 6-Fam or Tet fluorescent dye (Applied Biosystems, Foster City, CA). I amplified approximately half of my samples using 6-Fam as the label for both loci, and the remaining samples using Tet for both loci. Because loci were different sizes, they could be distinguished even if they were the same color and were co-loaded (loaded into the same gel lane for separation by electrophoresis and subsequent analysis). Labeling each primer set with each of two different dyes allowed co-loading of two samples and two loci to reduce per-lane analysis costs. Microsatellites were analyzed on an ABI 377 automated sequencer at the Iowa State University Sequencing Facility (Ames, Iowa). Allele sizes were assigned using Genotyper and Genescan Analysis software (Perkin-Elmer Corporation, Foster City, CA).

Data analysis

To delineate the range of sizes within which a given allele fell, I constructed cumulative frequency diagrams of raw allele scores for all individuals and for each dye for loci Ccar176 and CCM2 (Figs. 5–6). I binned all raw allele scores into named allele categories (i.e., an allele originally scored as 116.85 would be binned as allele 117 if the range of allele 117 was from 116.56 to 117.47). I eliminated eight individuals and one clutch from the analysis because they exhibited one or more of the following problems: A) individuals with raw scores for both alleles that fell outside of the normal allele size range, B) maternal alleles were lacking in an offspring, and the pattern of maternal alleles could not be explained by the presence of null allele(s) in the mother, C) more than two putative alleles were present in the maternal genotype. D) In clutches analyzed at four

loci, if an "extra" paternal allele indicating a second father appeared in only one hatchling at one locus, this allele was classified as a mutation (see Table 1). "Extra" paternal alleles are alleles which cannot be accounted for if only one mother and one father contributed to a clutch, and there was no mutation. In a clutch where one father was homozygous at a given locus, the second paternal allele would be extra, and in a clutch where one father was heterozygous at a given locus, the third observed allele would be extra. I assigned null alleles detected by parent-offspring genotype mismatches an arbitrary number (allele size of 100) and treated them like any other allele for analysis.

I assumed that clutches with more than two paternal alleles represented offspring from a mating between one female and two male loggerhead turtles. If more than four paternal alleles were present, I designated the clutch as having a minimum of three fathers. No clutches had more than five paternal alleles. Clutches that I determined had three fathers often had fewer than five alleles, but in these cases the distribution of alleles across loci could not be attributed to just two fathers.

I analyzed parental genotype and allele frequencies with Fisher's exact test as implemented in GENEPOP (Raymond and Rousset, 1995) to determine if: 1) maternal allele frequencies were in Hardy-Weinberg equilibrium, and 2) maternal allele and genotype frequencies differed between sampled locations. I calculated probabilities of detecting multiple fathers within a clutch with a single locus (d) and across multiple loci (D) as described in Westneat *et al.* (1987). Data from mothers from northern and southern sites were pooled for tests of deviation from Hardy-Weinberg Equilibrium (HWE) and linkage disequilibrium. Loci Ei8 and Cc117 were not in HWE, and were not used for parentage analysis. Remaining pairs of loci were tested for linkage disequilibrium using a

Markov chain method as implemented in Genepop. I genotyped offspring from all 70 clutches at Ccar176 and CCM2, and half of the clutches were also genotyped at Cc141 and Cc7.

RESULTS

Microsatellite analysis

Of the 150 nests originally marked in this project, 88 nests were located again, caged, and produced hatchlings. I chose 70 nests for analysis because they each had at least 10 sampled offspring. The 683 hatchlings from these nests yielded DNA of sufficient quantity and quality to amplify at least one locus. DNA from all 150 mothers amplified for at least two of the six tested loci, with the vast majority of maternal and hatchling samples amplifying at all loci (see Appendix B for maternal genotypes). Mothers from northern and southern sites did not differ significantly in allele distribution ($P = 0.06$ for Cc141, $P = 0.72$ for Cc7, $P = 0.22$ for Ei8, $P = 0.27$ for Cc117, $P = 0.18$ for Ccar176, and $P = 0.76$ for CCM2; Combined tests $X^2 = 12.97$, d.f. = 10, $P = 0.23$), and were pooled for subsequent tests. Loci Ei8 and Cc117 were not in HWE, due to heterozygote excess ($P < 0.001$ and $P = 0.01$, respectively). These loci were not used in further population analyses. The remaining loci were all in HWE ($P = 0.23$ for Cc141, $P = 0.30$ for Cc7, $P = 0.81$ for Ccar176, and $P = 0.21$ for CCM2; see Fig. 7 for allele frequency distributions). Linkage disequilibrium was not detected for any pair of loci (Table 2). Loci had 10–20 alleles and varied in heterozygosity from 0.73 to 0.90. The probability of detecting multiple fathers with a single locus (d) varied from 0.55 to 0.73 (Fig. 7). The probability (D) of detecting multiple fathers with both CCM2 and Ccar176 was 0.86, and for all four loci, D = 0.96.

Only two hatchlings were excluded from analysis due to the reason in category A (see text above and Table 1); I eliminated them from the analysis because they were unlikely to be due the normal stepwise mutations assumed to be operative for polymorphic microsatellites, and did not follow Mendelian patterns of inheritance. For example, mother 108 had alleles with raw scores of 117.5 and 127.1 at locus Ccar176, and 184.0 and 186.1 at locus CCM2. One offspring (hatchling 108f) exhibited only allele 125.2 at locus Ccar176, and had repeatably amplifiable peaks at 185.2 and 213.9 for locus CCM2. I excluded 108f from further analysis because it had no maternal alleles at locus Ccar176, and both alleles at CCM2 fell outside the normal size ranges of adjacent alleles for that locus (see Fig. 6). Offspring that did not exhibit any maternal alleles (category B) were also excluded from analysis if the parent-offspring genotype mismatch could not be attributed to the presence of a maternal null allele. These offspring were excluded because they were assumed to either be offspring from the adjacent nest of a different mother, or their genotype resulted from mutation. Only one clutch exhibited more than two maternal alleles (case C). Female 72 had three alleles at both CCM2 and Cc141. In addition to the mother having three alleles, the clutch exhibited many other unusual attributes: hatchlings 72j and 72m had no maternal alleles at CCM2, and none of the remaining hatchlings had three alleles, but each had at least one of the mother's three alleles at these loci. The extra alleles might be due to chromosomal anomalies; hatchlings without maternal alleles could be from a different clutch or their genotypes arose from mutation. At locus Ccar176, this clutch exhibited parent/offspring genotype mismatches that were consistent with the mother being heterozygous for a null allele. In any case, it

was impossible to assign paternal alleles, so clutch 72 was discarded from further analysis.

Locus CCM2, a dinucleotide repeat that usually yielded alleles with even-numbered sizes, had an odd allele that occurred at low frequency (Fig. 6). Females 42, 83, and 135 each had allele 197 at this locus (I had no offspring data for them), and female 122 had a genotype of 196/197, with three of her nine scored offspring receiving allele 197. Individuals with this odd allele were analyzed with the rest of the data set. Ccar176 and CCM2 both exhibited evidence for null alleles at low frequency.

Multiple Paternity

Based on criteria outlined above, 22 clutches had at least two fathers, and six had at least three fathers. Examples of clutches with 1, 2, and 3 evident fathers are detailed in Figures 8–10. Paternal genotypes could not be assigned in many cases where more than one father was present because there were multiple possible solutions to the problem of assigning alleles to specific fathers (Figs. 8–10). Figure 8 illustrates four clutches representative of those that likely had only one father. Paternal genotypes were assigned with reasonable confidence in the two-father clutches 35, 52, and 99 (Fig. 10). In clutch 35, one father had the genotype 208/216:127/135:178/188:190/208 (Cc141:Ccar176:CCM2:Cc7), while the other (represented by only one sampled hatchling) had 214/?:141/?:186/?:184? (question marks represent either the second allele at a homozygous locus or an unsampled paternal allele). The father of six offspring assayed in clutch 52 had the genotype 198/198:117/117:186/194:210/210, and the remaining two offspring were fathered by a male with 200/?:133/?:188/197:186/?. Clutch

99's two fathers shared two alleles at the two assayed loci. Paternal genotypes were 117/127:188/196 and 127/139:186/188 (Ccar176:CCM2). In clutches with a minimum of three fathers, paternal genotypes were not as easy to determine (Fig. 10). One solution for clutch 92 had father A as 117/135:178/188, father B 127/?:178/192, and father C 133/?:186/? (Ccar176:CCM2). Another possibility was father A as 117/?:178/188, B as 127/?:178/192, and C as 133/135:186/188. A third possibility was father A as 117/127:178/?, B as 133/135:186/188, and C as 117/127:188/192. Other solutions were possible, and likewise there were multiple solutions to clutches 28, 104, and the remaining three-father clutches. LAMP [Likelihood Analysis of Multiple Paternity, a maximum-likelihood program detailed in Kichler *et al.* (1999)] could not be used to determine most likely number of fathers and paternal genotypes because it did not consider the possibility of clutches with more than two fathers.

DISCUSSION

Distance-related population structure was not detected between female loggerheads nesting in the northern and southern sites of Melbourne Beach, FL. Multiple paternity, however, occurs commonly in the nests of Melbourne Beach loggerheads. Thirty-one percent of all nests showed multiple fathers, and almost 10% of nests had three or more fathers—more than have previously been reported for any sea turtle species.

Allele frequencies at the northern and southern sites were not significantly different from each other. This was not surprising, as loggerheads are less site-specific than green turtles at this location (D. Bagley, pers. comm.), and green turtles did not exhibit distance-related structure on this beach (Peare and Parker, 1996). The geographic scale in which population structure was evident in Mediterranean loggerheads was larger than that examined in this study. The mtDNA structure in the Mediterranean may also be a remnant of recent colonization (<12000 y ago) by two different matrilines after the last glacial period, and philopatry may be slowing the decay of founder-induced structure (Schroth *et al.*, 1996).

The use of molecular techniques to discover multiple paternity is important in elucidating the basic biology of this little-known species. The mating habits of loggerhead turtles are not as well-studied as those of green turtles, and scientists are only beginning to understand the habits of sea turtles away from the nesting beach, where they spend the

vast majority of their lives. Researchers have sighted isolated mounted pairs of loggerheads, and incidental observations by fishermen in Australian waters indicate that loggerheads may participate in mating aggregations similar to those observed in green turtles (Limpus *et al.*, 1984). Seasonal changes in testosterone titers in loggerheads are also similar to those of green turtles, and this, combined with aerial survey data on seasonal abundance, suggest that the reproductive ecology of these species is similar (Wibbels *et al.*, 1987a). Male green turtles in mating aggregations are indiscriminant, attempting copulation with other males, crude decoys, and even hapless humans (Booth and Peters, 1972; Bustard, 1972; Ehrhardt, 1995). Male green turtles mount multiple females, but these mountings do not invariably result in fertilization, and females do not always allow males to mount them (Booth and Peters, 1972; Limpus *et al.*, 1984; Dodd, 1988). Mating green turtles are often accompanied by "escort" males which attempt to dislodge the mounted male (Caldwell, 1959; Booth and Peters, 1972; Limpus *et al.*, 1984). Although mating aggregations of Australian loggerheads, including "courting groups," have been reported, they do not seem to be the rule (Limpus, 1985), and recent aerial observations of mating loggerheads in the southeastern U. S. reported only solitary courting pairs (Frick *et al.*, 2000). This is a notable absence, since east Florida hosts more than 70,000 nests per year (Anonymous, 2000). Aggregations and competition for females may be reduced in the Melbourne Beach population because of a highly female-biased sex ratio. Hatchlings leaving beaches at Cape Canaveral, FL (40 km north of my study site) were 93% female due to temperature-dependent sex determination and the feminizing effects of warm sands (Mrosovsky and Provancha, 1989). Sex ratios of juvenile loggerheads captured at Cape Canaveral were less skewed (63% female; Wibbels

et al., 1987b), but these animals were probably from rookeries in North and South Carolina as well as Florida, and the more northern (cooler) rookeries likely contributed a higher proportion of males. The sex ratio of breeding adults at Melbourne Beach was unknown, but if male loggerheads are philopatric like Australian green turtles (FitzSimmons *et al.*, 1997), and survivorship of male and female hatchlings were the same, then female turtles should far outnumber male turtles on the breeding grounds (even if male turtles had shorter remigration intervals). If there were plenty of females, male turtles should not expend energy attempting to dislodge mounted competitors, but instead mate serially with available females. The lack of mating aggregations and escort males may also be the norm for loggerheads, as most matings observed by Limpus (1985) were solitary pairs, and he reported only one aggregation (near Sandy Cape in southern Queensland).

Despite frenzied mating attempts, Australian green sea turtles show limited multiple paternity, perhaps because of sperm competition (FitzSimmons, 1998). Female sea turtles mate at the beginning of the nesting season and store sperm to fertilize the clutches for that season, allowing ample opportunity for sperm mixing and competition (Miller, 1997; FitzSimmons, 1998). Minisatellite data indicates that green turtle nests at Tortuguero, Costa Rica have multiple paternity, but small sample sizes and ambiguity of the genetic markers make it difficult to estimate the frequency of multiply-sired nests (Parker *et al.*, 1996). Allozyme data indicates that multiple paternity is common in Australian loggerheads (33% of clutches examined), but because of the low level of detected polymorphisms, this inference is based on deviations from expected Mendelian patterns of inheritance; in only one clutch was multiple paternity verified by the presence of an

"extra" paternal allele (Harry and Briscoe, 1988). In contrast to Australian green turtles, loggerhead clutches from Melbourne Beach had a high incidence (at least 31%) of multiple paternity, second only that of Kemp's ridleys (≥58%; Kichler *et al.*, 1999). This study shows, for the first time, strong evidence for the genetic contributions of more than two fathers to a clutch. There are doubtless fathers that remain undetected, either because their offspring were not sampled [10 eggs were sampled from clutches that average about 113 eggs (Van Buskirk and Crowder, 1994)], or because they shared alleles with other fathers. The value of 31% is a conservative estimate of the number of multiply-fathered clutches.

The advantages of multiple matings are many. Both male and female turtles expend considerable energy migrating from feeding to mating and nesting grounds, producing sperm and ova, and mating. Female turtles' energy investment in eggs is much larger than males' investment in sperm, and females also must survive the risky and energetically costly process of nesting several times. Because female turtles make such a high investment in producing and laying eggs, they may mate more than once to bet hedge (Watson, 1991; Watson, 1998), upgrade male quality from earlier matings (Birkhead *et al.*, 1993; Evans and Magurran, 2000), avoid inbreeding (Madsen *et al.*, 1992; Stockley *et al.*, 1993), avoid genetic incompatibility (Zeh and Zeh, 1996; Newcomer *et al.*, 1999; Vala *et al.*, 2000), ensure their eggs are fertilized with the most competitive sperm, and/or increase the genetic diversity of their offspring (Smith, 1984; Watson, 1991; Madsen *et al.*, 1992; Baer and Schmid-Hempel, 1999). All of these hypotheses assume that male quality varies, and all but the last of them assume that females cannot initially make an accurate assessment of intrinsic male quality.

Female turtles may bet-hedge or upgrade to compensate for possible poor mate choice, though the benefits of bet-hedging are thought to be small in large populations like the one at Melbourne Beach (Yasui, 1998). Because of the skewed sex ratio in this population, it may be that females choose to mate with the first male that they encounter to ensure fertilization, and then they can upgrade if they happen upon a better male later on. Though males may be in demand off Melbourne Beach, and female (and possibly male) sea turtles are philopatric, it is unlikely that female loggerheads mate to avoid inbreeding. Mortality is very high for young sea turtles, and it is doubtful that mature animals returning to the natal beach decades later would mate with close kin. Avoidance of genetic incompatibility and selection of competitive sperm are both plausible hypotheses for maintaining polyandry in sea turtles. It is unlikely that a female would be able to detect accurately whether a potential mate was genetically compatible or not, and fitness traits may be similarly difficult to assess. Multiple mating and post-copulatory paternity biasing, then, may be a female's way of obtaining sperm with a competitive edge and avoiding clutch loss due to genetic incompatibility.

Among all of these arguments for polyandry, the genetic diversity hypothesis is particularly attractive because of its specific relevance to sea turtles' life history patterns. Though females are philopatric, they certainly are not without error in their homing ability. If nesting females had always been accurate in locating their natal beach, it would almost certainly have meant the demise of the species, for they would not be able to colonize new nesting sites as beaches came and went throughout the millennia (Bowen *et al.*, 1989; Bowen *et al.*, 1992). These wayward, polyandrous, gravid females who find their way to new nesting beaches carry with them much more of the diversity of their

natal rookery than they would had they mated with only one male. This is particularly important in light of the theory that new nesting beaches may be colonized by as few as one or two gravid females, which may have led to the low nucleotide diversity seen within surveyed nesting populations, while maintaining shallow population structure between rookeries (Bowen *et al.*, 1992).

Whichever mechanism is operating to maintain polyandry in loggerheads, there is increasingly compelling evidence across taxa that sperm-competition and post-copulatory female choice can bias paternity in the offspring of polyandrous females, and that their offspring gain fitness benefits (Birkhead *et al.*, 1993; LaMunyon and Eisner, 1993; Olsson *et al.*, 1996; Zeh *et al.*, 1998; Evans and Magurran, 2000; also see Zeh and Zeh, 1996 and Jennions and Petrie, 2000 for reviews). In one study, bumble-bee colonies produced by queens inseminated with high-diversity sperm had fewer parasites and greater reproductive success than did colonies produced by queens who were inseminated with low-diversity sperm (Baer and Schmid-Hempel, 1999). Spencer *et al.* (1998) found that polyandrous rock wallabies whose consorts fathered some of their offspring had a higher probability of raising their young to independence than rock wallabies who either were monandrous or whose young were all due to extra-pair copulation. Female Swedish adders who mated multiply were shown to have higher offspring viability than those who were monandrous (Madsen *et al.*, 1992). And the offspring of female sierra dome spiders who mated multiply had higher offspring growth rates and attained larger sizes after emergence from their natal cocoons (Watson, 1998). Whether these benefits were hypothesized to be due to mate choice, genetic diversity, or avoidance of genetic incompatibility or inbreeding, the positive effects of polyandry on the offspring were

demonstrable, and there is no reason to suspect that loggerheads would not likewise benefit from multiple matings.

Unfortunately, I could not unequivocally assign paternal genotypes, so maternal and paternal allele frequencies could not be compared to determine if fathers and mothers represented one population. Likewise, I could not address the possibility of unequal paternal contribution.

In addition to the positive effects polyandry should have on offspring and population fitness, it should also increase N_e, though the effect on the Melbourne Beach rookery will be small because of the large size of the mating population (Sugg and Chesser, 1994). Although the increase in N_e may be negligible in a large population such as that on Melbourne Beach, the effect may be significant in very small (or depleted) populations. Such remnant nesting populations, the survivors of overharvest, are the focus of most conservation efforts dedicated to sea turtles. Microsatellites have provided insight into the cryptic world of sea turtles and their mating behavior, and opened new avenues for questions and discussion. With development of more rigorous statistical methodologies, researchers can address the questions posed by this study.

CONCLUSIONS

All of the microsatellite markers I used were highly polymorphic, and probabilities of detecting multiple paternity were high whether I used two (D = 0.86) or four (D = 0.96) markers. Despite the confounding effects of mutations (which contributed both length variability and null alleles), microsatellites unequivocally showed that nesting loggerheads at Melbourne Beach are inseminated by multiple males, and confirm the value of multiple loci in studies of parentage. The markers also showed that Melbourne Beach loggerheads did not show distance-related structure on a fine scale, as observed for Mediterranean loggerheads (Schroth *et al.*, 1996) and Costa Rican green turtles (Peare and Parker, 1996). Questions concerning possible unequal paternal contribution and whether mating males and females shared the same allele frequencies could not be addressed because of the difficulty of assigning paternal genotypes.

Although the effects of multiple matings on N_e may be negligible in this very large population, the occurrence of multiple paternity here suggests it occurs frequently, and may help to preserve genetic diversity in smaller populations of loggerheads. Once paternal genotypes are statistically resolved, researchers can answer questions of unequal male contribution and panmixis, as well as investigate the effects of each father's contribution to fitness.

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Table 1. Samples excluded from further analysis. The justification is discussed in more detail in the text.

Sample number	Locus	Reason discarded
108g	CCM2	A) Alleles outside normal range
134d	CCM2	A) Alleles outside normal range
40a	Ei8, Cc141	B) No maternal alleles
55f	Cc141	B) No maternal alleles
72j	CCM2	B) No maternal alleles
108g	Ccar176	B) No maternal alleles
141q	Ccar176	B) No maternal alleles
141v	Ccar176	B) No maternal alleles
Clutch 72	CCM2, Cc141	C) Three maternal alleles
45b	CCM2	D) “Extra” allele due to mutation
34c	CCM2	D) “Extra” allele due to mutation

Table 2. P-values and standard errors of the mean for results of genotypic disequilibrium test between loci Ccar176, CCM2, Cc141, and Cc7.

Locus 1	Locus 2	X^2	df	P	S.E.
Ccar176	CCM2	1.65	2	0.43	0.05
Ccar176	Cc141	3.29	2	0.19	0.04
CCM2	Cc141	0.66	2	0.72	0.04
Ccar176	Cc7	0.00	2	1.00	0.00
CCM2	Cc7	2.41	2	0.30	0.04
Cc141	Cc7	1.98	2	0.37	0.05

Figure 1. Nest cages were about 1 m high, made of 1 cm mesh hardware cloth, and had removable chicken wire lids. A seaward door was left open during the day to allow hatchlings emerging in daylight to escape.

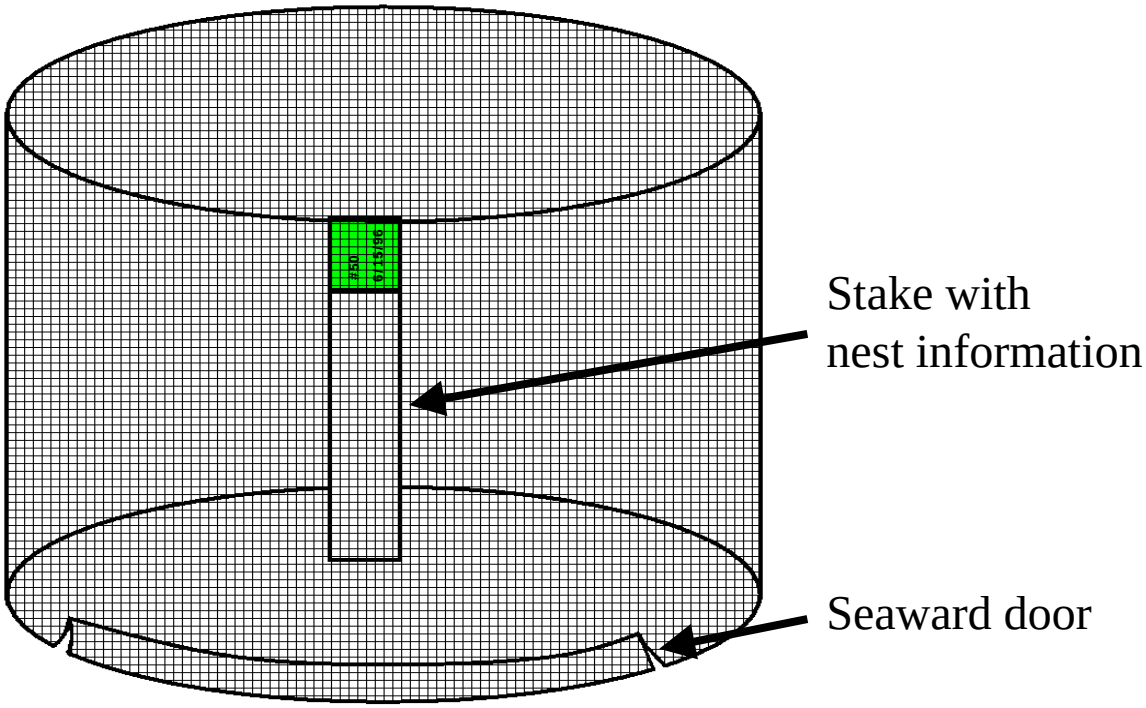

Stake with
nest information
Seaward door

Figure 2. Ventral view of hatchling, showing approximate location and relative size of shell biopsy. Diagram modified from Arenas *et al.*, 1998.

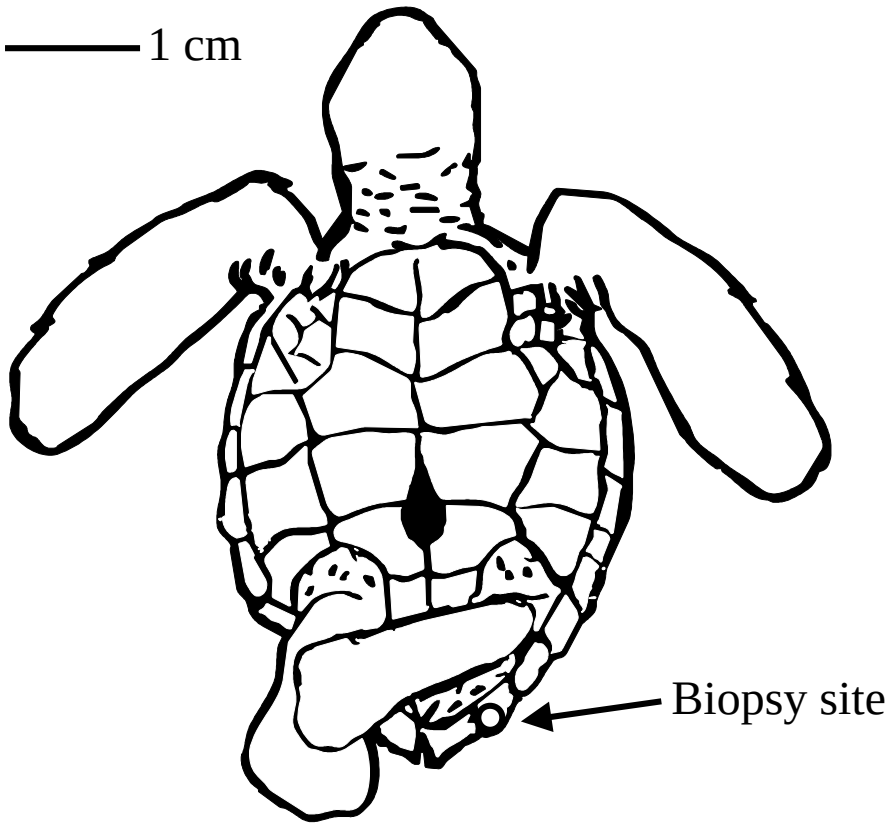

1 cm
Biopsy site

Figure 3. Steps involved in the construction of a DNA library for microsatellite enrichment (based on Armour *et al.*, 1994, and modified by the University of Florida Education core).

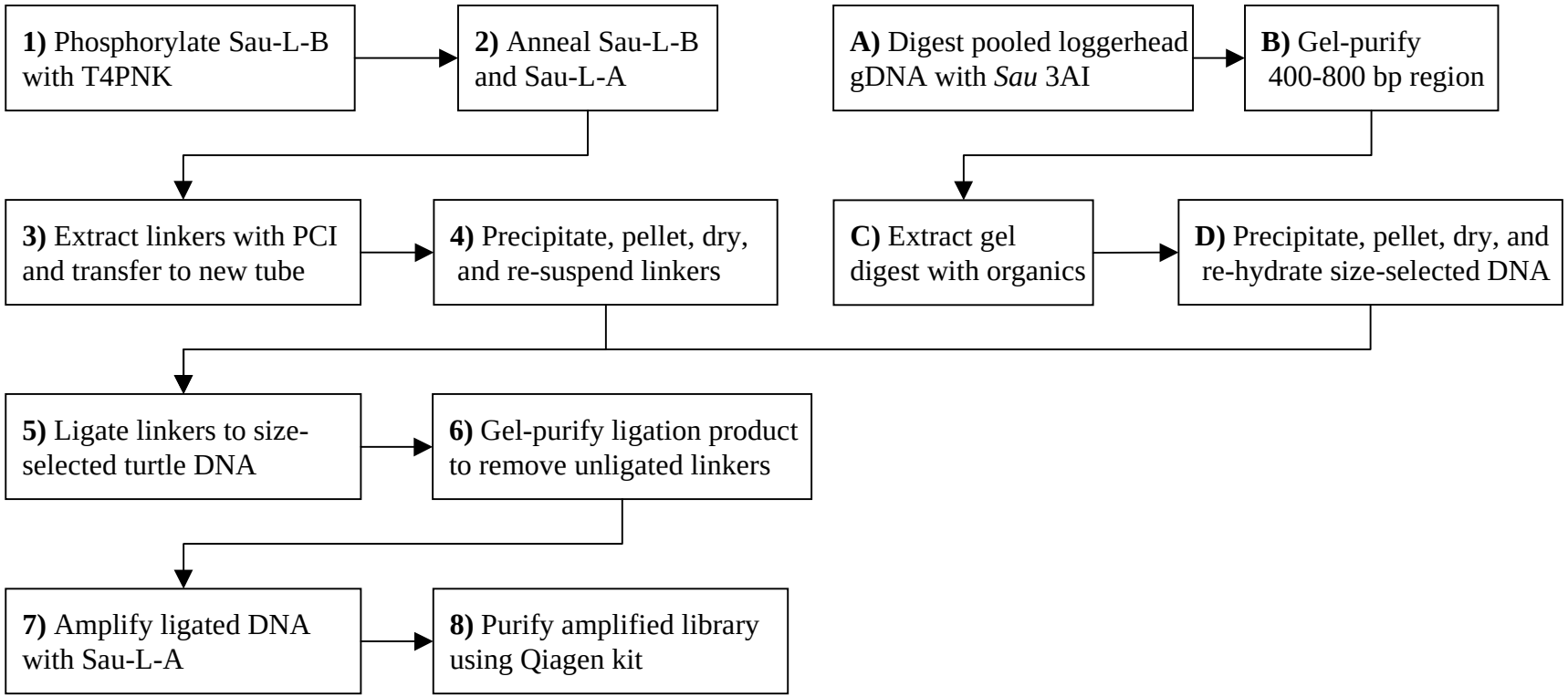

1) Phosphorylate Sau-L-B with T4PNK
2) Anneal Sau-L-B and Sau-L-A
A) Digest pooled loggerhead gDNA with *Sau* 3AI
B) Gel-purify 400-800 bp region
3) Extract linkers with PCI and transfer to new tube
4) Precipitate, pellet, dry, and re-suspend linkers
C) Extract gel digest with organics
D) Precipitate, pellet, dry, and re-hydrate size-selected DNA
5) Ligate linkers to size-selected turtle DNA
6) Gel-purify ligation product to remove unligated linkers
7) Amplify ligated DNA with Sau-L-A
8) Purify amplified library using Qiagen kit

Figure 4. Electropherograms of Ccar176 and Ccar199, polymorphic microsatellites developed for this study. Ccar176 amplified consistently and with little stutter. Ccar199 stuttered too badly to score, and was not used. Both examples above are heterozygotes.

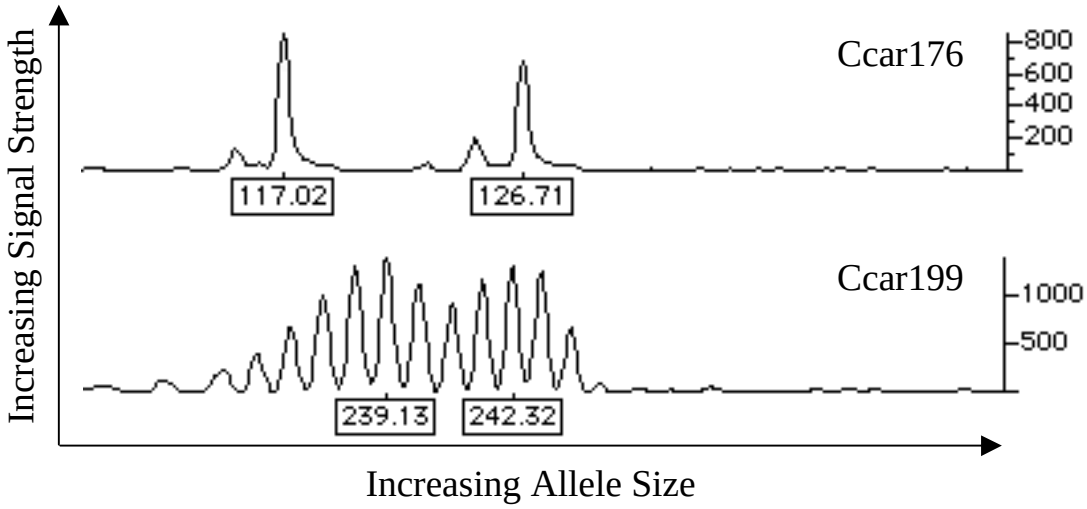

Increasing Signal Strength
Ccar176
800
600
400
200
117.02
126.71
Ccar199
1000
500
239.13
242.32
Increasing Allele Size

Figure 5. Cumulative frequency of all alleles scored at Tet- and 6-Fam–labeled locus Ccar176.

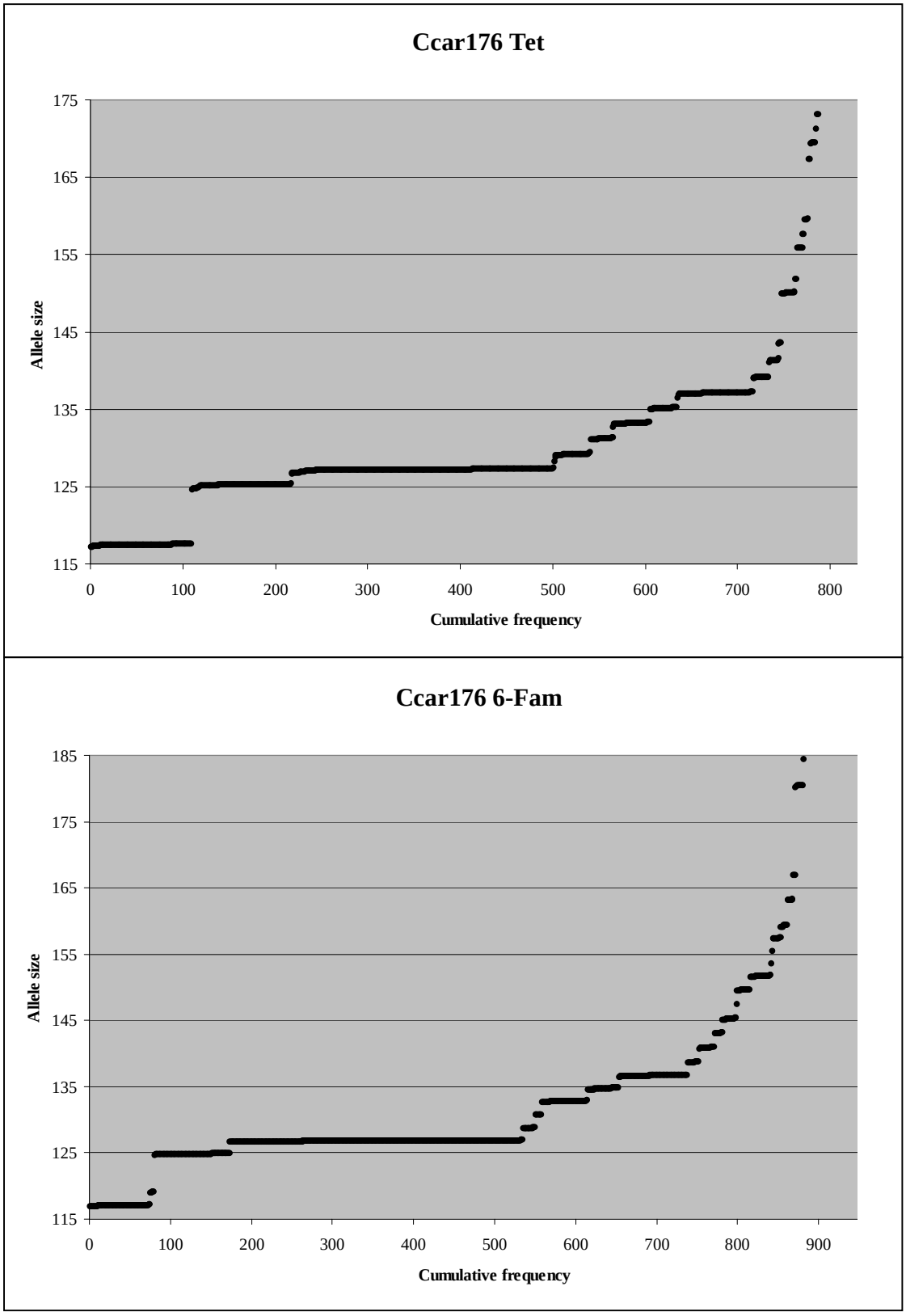

Ccar176 Tet
175
165
155
145
135
125
115
Allele size
0
100
200
300
400
500
600
700
800
Cumulative frequency
Ccar176 6-Fam
185
175
165
155
145
135
125
115
Allele size
0
100
200
300
400
500
600
700
800
900
Cumulative frequency

Figure 6. Cumulative frequency of all alleles scored at Tet- and 6-Fam–labeled locus CCM2. The two alleles marked with open circles on the CCM2 Tet diagram were from hatchling 108g; both were outside the normal range for adjacent alleles. This hatchling also had no maternal allele at locus Ccar176, and was excluded from further analysis due to non-Mendelian patterns of inheritance. This even-scored locus also had an odd allele (197), which occurred at low frequency, but followed a Mendelian pattern of inheritance.

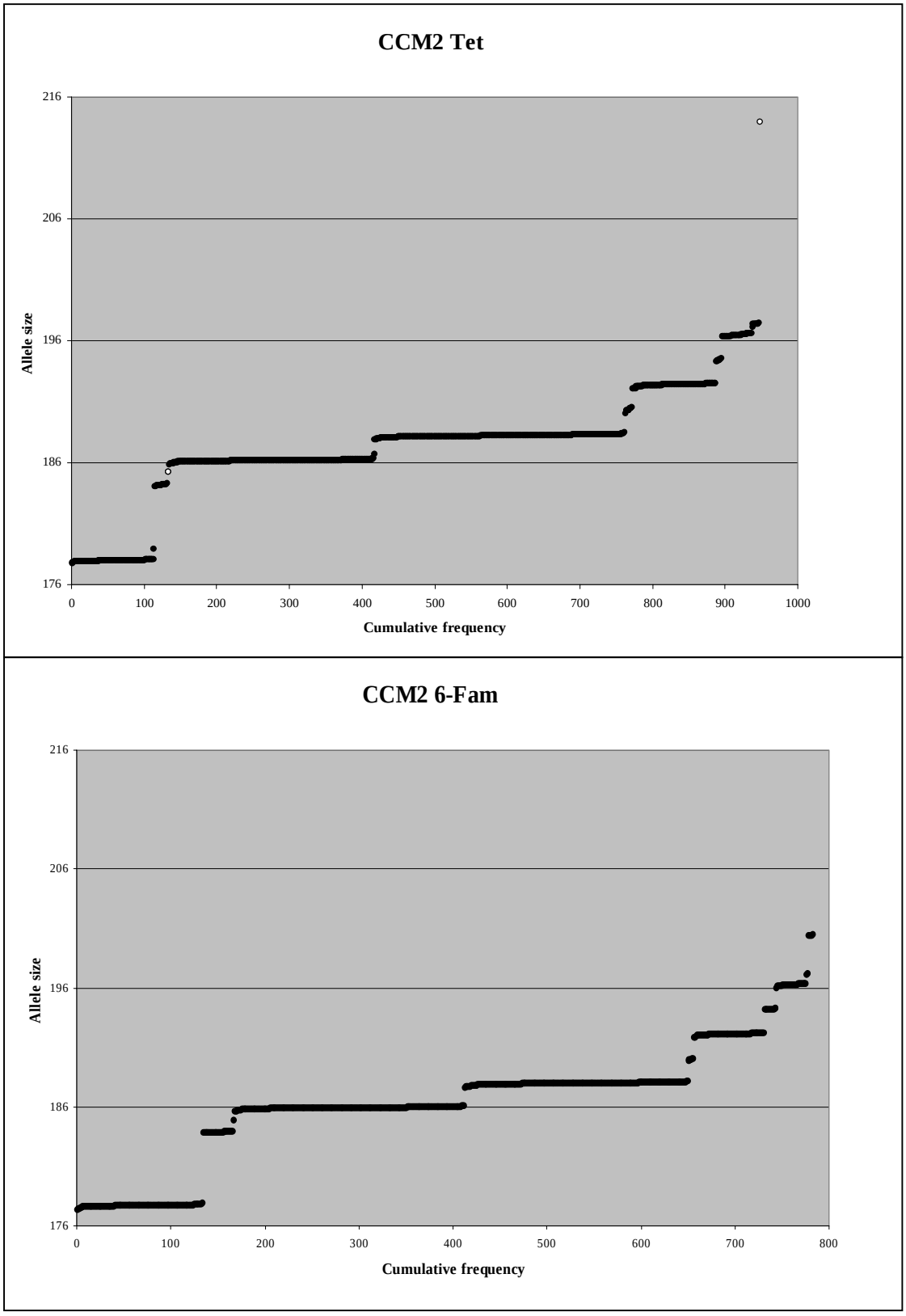

CCM2 Tet
216
206
196
186
176
Allele size
0
100
200
300
400
500
600
700
800
900
1000
Cumulative frequency
CCM2 6-Fam
216
206
196
186
176
Allele size
0
100
200
300
400
500
600
700
800
Cumulative frequency

Figure 7. Maternal allele frequencies, observed heterozygosities (H), and the probabilities of detecting multiple fathers within a clutch (d) for four microsatellite loci.

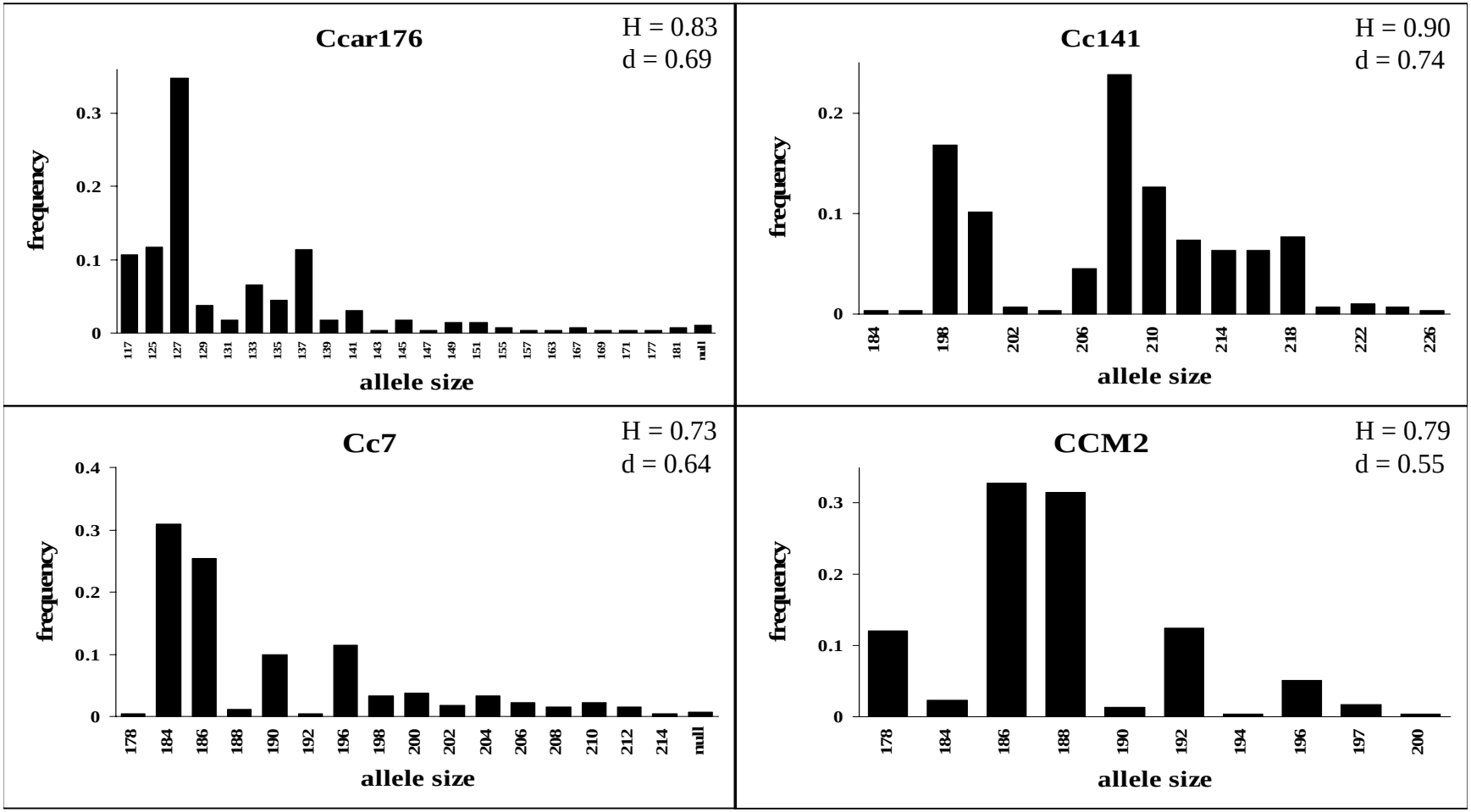

Ccar176
H = 0.83
d = 0.69
frequency
0.3
0.2
0.1
0
117 125 127 129 131 133 135 137 139 141 143 145 147 149 151 155 157 163 167 169 171 177 181 null
allele size
Cc141
H = 0.90
d = 0.74
frequency
0.2
0.1
0
184 198 202 206 210 214 218 222 226
allele size
Cc7
H = 0.73
d = 0.64
frequency
0.4
0.3
0.2
0.1
0
178 184 186 188 190 192 196 198 200 202 204 206 208 210 212 214 null
allele size
CCM2
H = 0.79
d = 0.55
frequency
0.3
0.2
0.1
0
178 184 186 188 190 192 194 196 197 200
allele size

Figure 8. Schematic representation of four clutches assumed to have only one father. Numbers represent sizes of paternal alleles present in the offspring, and each vertical path from Cc141 to Cc7 represents a hatchling's paternally inherited multi-locus haplotype. For example, one hatchling from clutch 1 inherited 206:127:186:190 (Cc141:Ccar176:CCM2:Cc7), and another hatchling inherited 206:137:186:186.

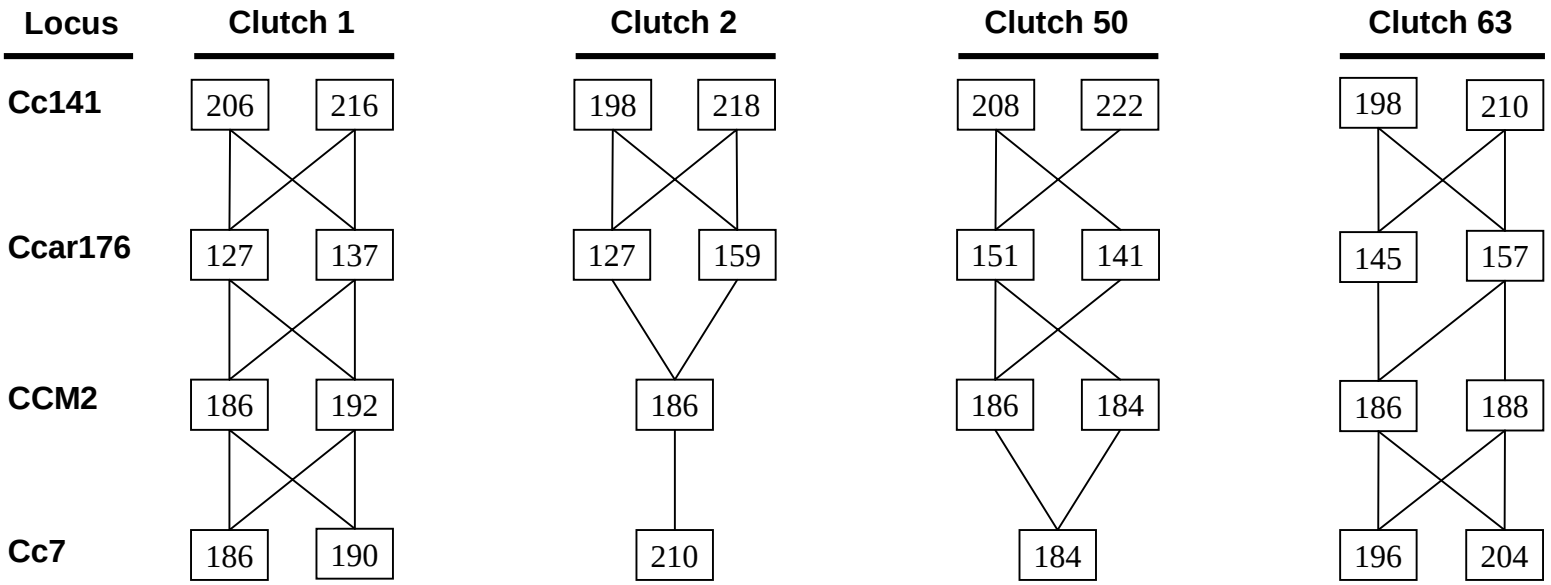

Locus
Clutch 1
Clutch 2
Clutch 50
Clutch 63
Cc141
Ccar176
CCM2
Cc7
206
216
127
137
186
192
186
190
198
218
127
159
186
210
208
222
151
141
186
184
184
198
210
145
157
186
188
196
204

Figure 9. Schematic representation of three clutches assumed to have at least two fathers. Numbers represent sizes of paternal alleles present in the offspring, and each vertical path from Cc141 to Cc7 (or from Ccar176 to CCM2 if data from other loci were not available) represents a hatchling's paternally-inherited multi-locus haplotype (see Fig. 8 for examples).

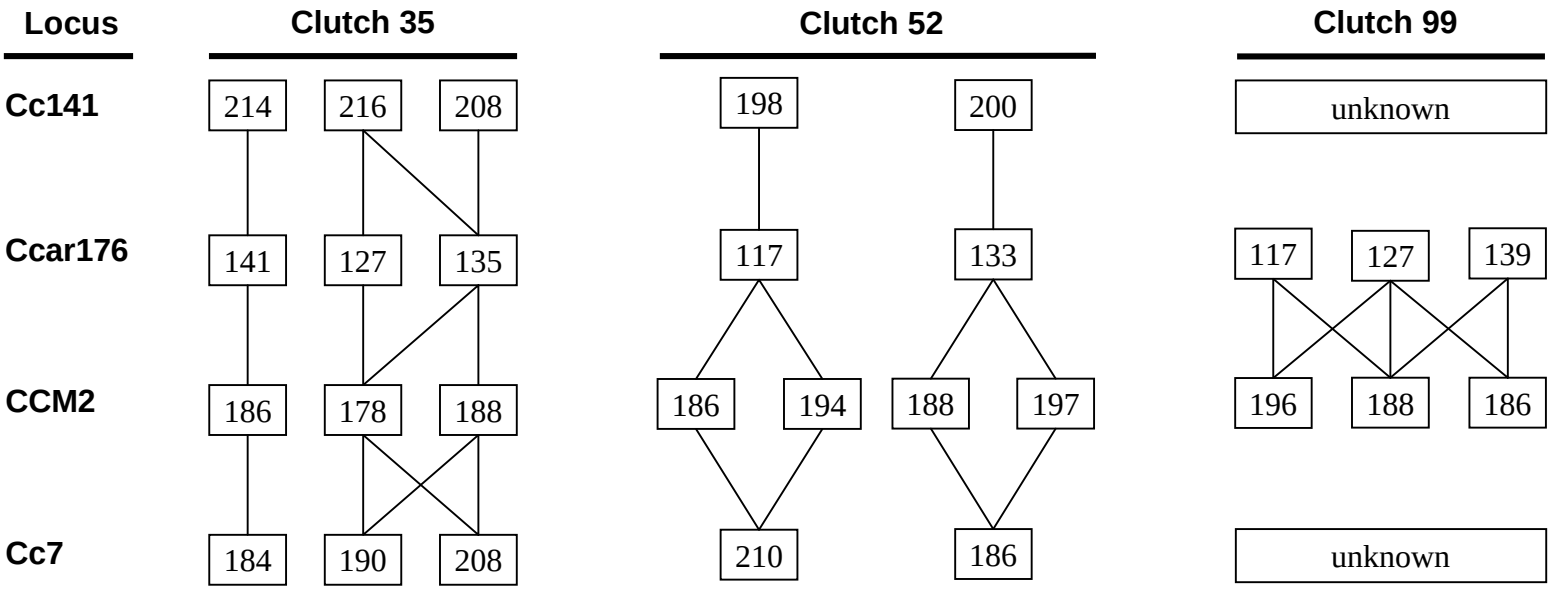
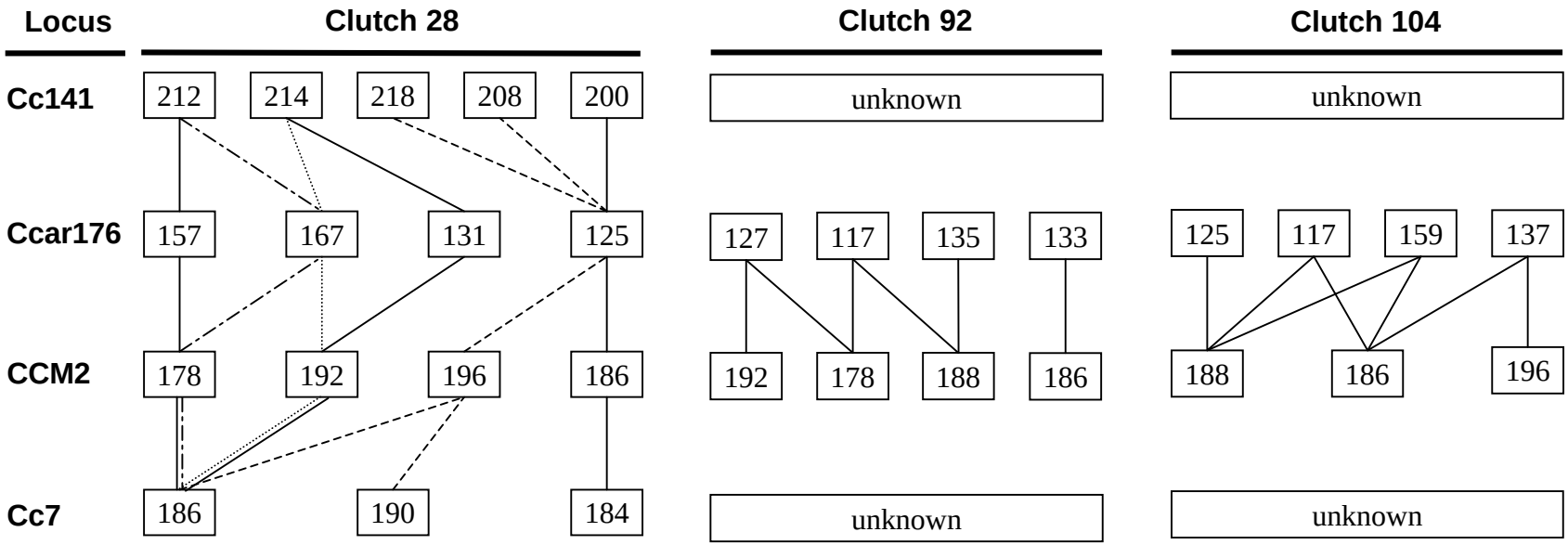

Locus
Clutch 35
Clutch 52
Clutch 99
Cc141
Ccar176
CCM2
Cc7
214
216
208
141
127
135
186
178
188
184
190
208
198
200
117
133
186
194
188
197
210
186
unknown
117
127
139
196
188
186
unknown

Figure 10. Schematic representation of three clutches assumed to each have at least 3 fathers. Numbers represent sizes of paternal alleles present in the offspring, and each vertical path Cc141 to Cc7 (or from Ccar176 to CCM2 if data from other loci are not available) represents a hatchling's paternally-inherited multi-locus haplotype (see Fig. 8 for examples). Different dashed lines represent combinations of alleles associated across loci in hatchlings. For example, Clutch 28 hatchlings with Cc141 allele 212 also had Ccar176 allele 157 or 167, CCM2 allele178 (but not CCM2 allele 192), and Cc7 allele 186.


Locus
Clutch 28
Clutch 92
Clutch 104
Cc141
Ccar176
CCM2
Cc7
212
214
218
208
200
157
167
131
125
178
192
196
186
186
190
184
unknown
127
117
135
133
192
178
188
186
unknown
unknown
125
117
159
137
188
186
196
unknown

APPENDIX A: PRIMER INFORMATION

Table A 1 Primer sequences, annealing temperatures (T_a), size of amplified product, type of repeat, and observed (H_o) and expected (H_e) heterozygosities of microsatellite loci characterized for this study. Lower-case bases at the 3′ end of some primer sequences represent additional non-templated bases of the PIGtail region (see text for explanation).

Locus	Primer sequence	T_a (°C)	Size (bp)	Repeat type	H_o	H_e
Ccar012	F: CCTTAGCATCCCAGGCTCTTG	57	228	$(CCT)_7$	0	
	R: gtttACAGCGGCACATGACAAATA					
Ccar041	F: gtttCACGACGAAGTGCCAGTAGA	59	145	$(AGG)_8$	0	
	R: CTTGCCACTCCACCAGATGC					
Ccar176	F: GGCTGGGTGTCCATAAAAGA	60	117–181	$(AC)_8$	0.83	0.83
	R: gtTTGATGCAGGAGTCACCAAG					
Ccar199	F: AGGTGGCTTTTCTTGCTCCT	60	169–219	$(TC)_{16}(AC)_{38}$	?	?
	R: gtTTCAAGCGTAACTGCCTTCC					
Ccar202	F: CTTGAGGACCTGCTCCAT	52	239	$(GT)_{11}$	0	
	R: CGGGTCACCATACTTAAATC					
Ccar216	F: TCAGAGACTCAGCCCAGGAT	52	171	$(GT)_{37}$ w/multiple	0	
	R: CCACAGACCTACTGCGTTCA			imperfections		
Ccar233	F: AGTAGACAGCAGAGGTGCCG	54	128	$(CA)_{23}$ w/multiple	0	
	R: AGGGCGTTCATAGCCTGTC			imperfections		
CCM2	F: gtttTGGCACTGGTGGAAT	58	185	$(CA)_{18}$	0.79	0.76
(Francisco)	R: TGACTCCCAAATACTGCT					

APPENDIX B: MATERNAL GENOTYPES

Table B 1 Multi-locus genotypes for all 150 nesting females. Numbers 1–75 are females from the northern site, and 76–150 are from the southern site. Not all females were assayed at Cc117 due to inconsistent amplification. Question marks indicate missing data or the second allele in a homozygous individual (when homozygosity could not be assigned with confidence).

Female #	Cc176	CCM2	Cc141	Cc7	Ei8	Cc117
1	125/125	178/188	218/208	200/184	203/189	257/245
2	127/135	178/188	220/214	196/184	197/189	257/245
3	117/135	178/192	212/210	210/184	197/183	251/245
4	117/137	186/188	210/208	206/186	199/189	253/251
5	133/141	186/192	218/208	186/186	189/187	251/251
6	127/145	188/196	210/198	186/184	201/187	257/245
7	125/127	188/188	200/200	202/190	201/199	257/245
8	127/181	186/188	216/208	186/184	187/189	257/251
9	127/127	186/192	218/198	184/184	189/175	251/245
10	129/137	186/188	210/208	204/184	199/189	251/245
11	127/127	178/188	218/198	206/184	199/197	251/245
12	125/127	188/188	208/198	190/186	201/189	257/245
13	125/181	186/186	220/210	198/186	201/199	257/245
14	127/127	186/186	214/200	212/196	203/189	257/257
15	127/135	188/188	210/208	196/190	189/187	251/245

Table B 1, continued.

Female #	Cc176	CCM2	Cc141	Cc7	Ei8	Cc117
16	127/151	186/188	212/212	184/184	195/189	257/245
17	127/129	178/186	208/200	196/184	201/189	257/251
18	125/145	186/186	208/200	196/184	201/199	251/245
19	125/129	186/186	208/200	184/184	201/189	259/251
20	117/133	188/188	208/208	196/196	201/189	245/245
21	127/141	178/186	216/200	186/186	199/197	247/247
22	117/127	186/192	212/200	202/186	201/195	251/245
23	117/141	188/190	210/198	184/184	201/199	257/247
24	127/127	186/188	216/198	186/184	201/199	257/253
25	117/117	192/192	210/206	196/184	201/197	251/245
26	127/127	186/186	218/198	190/184	203/199	251/245
27	125/127	178/186	216/198	190/190	201/189	257/247
28	133/137	188/196	218/208	210/198	201/189	245/237
29	117/133	186/186	216/210	186/186	201/187	251/245
30	127/137	186/192	208/200	200/186	201/183	251/245
31	127/137	188/192	218/198	184/184	201/197	257/245
32	127/133	184/188	212/198	192/186	201/187	251/245
33	127/137	184/186	214/206	204/204	189/187	251/245
34	127/127	186/194	218/200	212/206	201/187	251/245
35	125/127	178/196	222/198	208/184	201/187	251/245
36	127/139	186/188	208/200	208/184	189/187	251/251
37	141/163	186/192	216/212	206/184	201/183	257/251
38	131/133	186/188	214/206	186/186	201/187	245/241
39	135/147	186/188	218/210	196/184	201/189	257/221
40	135/149	184/186	216/208	190/186	201/199	257/?

Table B 1, continued.

Female #	Cc176	CCM2	Cc141	Cc7	Ei8	Cc117
41	137/157	186/186	200/200	186/186	201/195	257/251
42	127/137	188/197	214/200	204/184	201/189	251/251
43	127/145	178/188	198/198	?/184	?/189	?/?
44	127/127	186/192	208/200	188/184	201/189	257/251
45	127/143	?/?	210/198	196/186	203/187	255/249
46	127/139	178/178	208/198	186/184	201/197	255/247
47	117/127	186/192	212/208	206/198	201/195	245/?
48	129/133	188/192	214/208	186/178	201/199	251/245
49	117/127	188/190	218/208	186/184	201/189	251/241
50	125/null	186/192	210/210	198/190	189/189	245/245
51	125/131	186/192	212/198	196/null	201/199	247/245
52	127/135	178/188	198/198	186/184	195/189	257/?
53	129/141	188/192	200/198	?/?	?/189	?/245
54	127/127	186/196	?/?	?/?	?/187	251/245
55	133/151	186/192	216/204	210/190	?/?	?/245
56	127/145	188/190	200/198	?/?	197/183	251/245
57	135/null	178/178	214/198	186/184	201/197	251/245
58	127/155	188/188	216/210	?/?	201/187	251/247
59	117/137	186/188	208/198	?/?	?/187	?/?
60	127/133	188/196	200/198	?/?	?/?	?/?
61	?/?	178/188	218/208	?/?	?/187	?/?
62	127/137	188/200	200/198	186/186	201/199	?/?
63	127/127	184/186	210/208	186/186	?/?	257/251
64	117/125	186/188	208/198	?/?	187/187	257/245
65	127/137	178/196	210/?	184/null	201/189	257/245

Table B 1, continued.

Female #	Cc176	CCM2	Cc141	Cc7	Ei8	Cc117
66	127/137	178/196	?/?	?/?	?/?	?/245
67	125/127	186/192	210/200	186/184	201/197	257/247
68	127/137	184/188	208/?	186/184	195/187	257/251
69	125/141	186/188	208/198	186/184	?/?	?/?
70	127/127	178/186	210/208	?/?	?/?	?/245
71	125/127	186/188	210/208	196/190	?/?	?/245
72	127/null	178/186/188	218/210/198	204/186	201/199	?/?
73	127/127	178/186	210/198	?/?	?/?	257/251
74	133/145	188/192	210/208	184/184	201/?	?/?
75	117/127	186/188	208/?	186/184	?/?	251/?
76	133/137	186/192	210/208	200/184	?/?	?/?
77	117/125	188/190	210/208	196/186	201/199	?/?
78	117/135	188/188	214/206	210/186	?/187	?/?
79	125/137	186/188	208/198	184/184	201/187	257/?
80	129/137	186/192	224/198	196/186	?/189	257/?
81	127/149	178/188	210/208	184/184	201/189	251/245
82	?/?	186/188	210/208	208/184	197/189	257/245
83	129/133	186/197	216/208	196/196	201/?	257/245
84	117/137	188/192	226/198	190/188	199/197	259/245
85	117/127	186/192	214/208	208/184	201/189	257/245
86	127/137	186/196	208/208	202/186	?/?	251/245
87	127/155	188/196	224/214	200/188	201/189	257/251
88	127/137	188/188	214/214	190/184	197/193	257/251
89	125/125	186/186	192/184	184/184	201/189	257/245
90	127/127	186/188	218/208	196/184	201/187	245/245

Table B 1, continued.

Female #	Cc176	CCM2	Cc141	Cc7	Ei8	Cc117
91	127/129	186/186	218/216	186/184	189/175	257/249
92	117/127	186/186	210/210	186/184	201/197	257/251
93	133/137	188/188	210/?	204/202	189/173	257/251
94	117/127	178/186	198/198	200/186	195/189	251/247
95	?/?	188/188	214/208	196/184	189/?	?/251
96	127/135	186/192	?/?	196/186	?/?	?/?
97	129/133	186/196	224/198	184/184	189/?	257/245
98	133/137	186/192	218/198	206/200	201/189	257/251
99	127/137	178/188	212/208	190/184	201/?	259/251
100	125/139	188/192	210/202	196/196	197/189	257/245
101	137/137	186/186	208/206	204/198	203/189	257/251
102	127/137	188/188	212/208	196/184	201/183	257/249
103	117/117	178/186	214/212	196/186	201/195	257/245
104	117/125	188/188	222/214	204/186	199/189	251/?
105	125/135	186/192	216/208	200/184	187/175	251/?
106	127/141	188/188	212/210	196/190	?/?	257/251
107	117/137	186/186	212/208	186/184	203/189	253/245
108	117/127	184/186	200/198	190/186	195/?	?/?
109	125/125	186/188	218/208	184/184	189/177	257/251
110	127/139	186/188	194/?	?/?	?/?	?/?
111	127/139	186/188	216/202	190/186	203/197	249/245
112	133/137	186/196	218/208	186/184	189/175	253/251
113	125/127	188/188	212/200	190/184	201/189	251/?
114	117/127	178/188	218/214	198/198	197/189	257/245
115	127/127	178/188	208/198	190/186	201/195	245/245

Table B 1, continued.

Female #	Cc176	CCM2	Cc141	Cc7	Ei8	Cc117
116	125/127	186/192	216/208	200/186	201/189	?/251
117	127/167	178/188	212/198	190/186	199/189	253/251
118	127/169	178/188	212/198	190/186	?/?	253/251
119	137/167	188/196	208/206	212/198	201/189	251/247
120	117/117	178/196	208/206	186/184	201/189	251/245
121	000/000	186/188	212/198	202/184	201/?	?/?
122	117/141	196/197	216/200	214/184	205/189	?/?
123	127/135	178/196	198/198	198/186	201/187	?/?
124	127/127	178/188	208/200	200/184	?/?	?/?
125	117/131	178/186	208/200	184/184	?/?	?/?
126	127/149	188/192	210/?	186/184	?/?	?/?
127	125/127	186/192	208/198	184/184	201/?	?/?
128	149/171	188/192	208/200	?/196	197/191	?/?
129	125/133	178/186	218/208	190/184	197/187	?/?
130	127/131	188/192	208/208	196/190	189/?	?/?
131	129/133	186/192	212/210	186/184	201/189	?/?
132	?/?	186/188	212/210	196/184	201/189	?/?
133	125/127	186/188	222/208	196/184	?/?	?/?
134	125/127	178/186	210/208	186/184	201/189	?/?
135	127/135	192/197	216/206	196/190	?/?	?/?
136	137/141	186/196	208/206	200/190	201/189	?/?
137	117/127	186/188	216/212	186/184	201/189	?/?
138	127/133	188/188	218/208	196/184	199/189	?/?
139	125/127	186/186	216/198	210/184	199/187	?/?
140	127/135	188/192	218/206	212/?	201/187	?/?

Table B 1, continued.

Female #	Cc176	CCM2	Cc141	Cc7	Ei8	Cc117
141	131/149	188/192	200/198	190/184	197/187	?/?
142	129/137	186/192	210/200	212/186	203/189	?/?
143	125/125	186/188	214/198	210/190	201/189	?/?
144	127/177	186/186	212/206	186/184	203/189	?/?
145	137/137	178/184	214/206	186/186	201/189	?/?
146	117/127	186/188	218/206	186/186	201/187	?/?
147	125/149	178/188	208/198	204/186	201/189	?/?
148	127/137	186/188	210/200	186/186	201/189	?/?
149	127/127	188/192	208/208	196/186	201/189	?/?
150	127/127	186/188	208/198	190/184	201/187	?/?